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Patterns of cellular gene expression in swine macrophages infected with highly virulent classical swine fever virus strain Brescia

Manuel V. Borca[b], Ingigerdur Gudmundsdottir[a], Ignacio J. Fernández-Sainz[b], Lauren G. Holinka[b], Guillermo R. Risatti[a,*]

[a] Department of Pathobiology and Veterinary Science, University of Connecticut, Storrs, CT 06498-3089, United States
[b] Plum Island Animal Disease Center, Agricultural Research Service, United States Department of Agriculture, Greenport, NY 11944, United States

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ABSTRACT

Experimental exposure of swine to highly virulent classical swine fever virus (CSFV) strain Brescia causes an invariably fatal disease of all infected animals by 8–14 days post-infection. Host mechanisms involved in this severe outcome of infection have not been clearly established. To understand these mechanisms, we analyzed the response of primary cultured swine macrophages, a CSFV primary target cell, to infection with Brescia strain. Steady state levels of mRNA accumulation were assessed for 58 genes involved in modulation of the host immune response, at 24 and 48 h post-infection (hpi), by means of quantitative reverse transcription real-time PCR analysis (qrt-PCR). Eighteen genes showed altered expression upon infection with CSFV strain Brescia including: cytokines (IL-1α, IL-1β, IL-6, and IL-12p35); cytokine receptors (IL-2Rα, IL-12Rβ, and TGF-βIIIR); chemokines (IL-8, AMCF-1, AMCF-2, MCP-2, and RANTES); interferons (INFα and INFβ); and toll-like receptors (TLR3, TLR5, TLR9, and TLR10). Although these genes are associated with mechanisms of innate immune response and antiviral activity, their altered expression does not curtail CSFV Brescia growth kinetics and virus yield in swine macrophages. Data gathered here suggests that the observed gene expression profile might explain immunological and pathological changes associated with virulent CSFV infections.

1. Introduction

Classical swine fever (CSF) is a highly contagious disease of swine that can be either acute or chronic in nature (Van Oirschot, 1999). The etiological agent, CSF Virus (CSFV), is a small, enveloped virus with a positive, single-stranded RNA genome and, along with *Bovine Viral Diarrhea Virus* (BVDV) and *Border Disease Virus* (BDV), is classified as a member of the genus *Pestivirus* within the family *Flaviridae* (Becher et al., 2003). The 12.5 kb CSFV genome contains a single open reading frame which encodes a 4000-amino-acid polyprotein and ultimately yields 11–12 final cleavage products (NH_2-N^{pro}-C-E^{rns}-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH) through co- and post-translational processing of the polyprotein by cellular and viral proteases (Rice, 1996).

Virulence and host range phenotypes vary among CSFV isolates and between pestiviruses. Infection with highly virulent CSFV strains leads to death in infected animals, whereas isolates of moderate to low virulence induce a prolonged chronic disease (Van Oirschot, 1999). In addition, BVDV and BDV, while etiologic agents of diseases in bovine and ovine species, respectively, can also infect swine without inducing clinical disease (Van Oirschot, 1999). Despite availability of genomic sequences from CSFV of differing virulence phenotypes, the genetic basis of CSFV virulence in the natural host remains poorly understood (Van Oirschot, 1999).

Viral determinants of virulence have been identified (Mayer et al., 2004; Meyers et al., 1999; Moser et al., 2001; Risatti et al., 2005a,b, 2006, 2007a,b; Ruggli et al., 2005; Tratschin et al., 1998; van Gennip et al., 2002, 2004). Notably, all three viral glycoproteins have been associated with CSFV virulence (Meyers et al., 1999; Risatti et al., 2005a,b, 2006, 2007a,b; Sainz et al., 2008; van Gennip et al., 2004).

CSFV strain Brescia has a highly virulent pathotype. Infected animals die within 8–14 days after exposure. Brescia belongs to CSFV genotype 1 (Lowings et al., 1994) together with other virulent strains isolated before 1964 (Eystrup, Weybridge, ALD, Alfort/187) that presumably are no longer circulating among swine populations around the world.

Due to the consistent clinical outcome observed in infected swine, highly virulent CSFV strains provide a reliable method

* Corresponding author at: Department of Pathobiology and Veterinary Science, University of Connecticut, 61 N Eagleville Road, Unit 3089, Storrs, CT 06269-3089, United States. Tel.: +1 860 486 6150/9435; fax: +1 860 486 2794.
E-mail address: guillermo.risatti@uconn.edu (G.R. Risatti).

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for studying mechanisms underlying virulence, pathogenesis, and virus–host interactions. Here we have used the Brescia strain to study the response of swine macrophages, a CSFV primary target cell *in vivo*, upon infection. Cultured primary swine macrophages were infected with CSFV Brescia derived from an infectious cDNA clone, pBIC (Risatti et al., 2005a). Steady state level accumulation of mRNAs from 58 genes was measured by quantitative reverse transcription real-time polymerase chain reaction (qrt-PCR) at 24 and 48 h post-infection (hpi). Transcriptional activation of 18 genes was induced upon infection with CSFV Brescia including: cytokines (IL-1α, IL-1β, IL-6, and IL-12p35); cytokine receptors (IL-2Rα, IL-12Rβ, and TGF-βIIIR); chemokines (IL-8, AMCF-1, AMCF-2, MCP-2, and RANTES); interferons (INFα and INFβ); and toll-like receptors (TLR3, TLR5, TLR9, and TLR10) genes.

2. Materials and methods

2.1. Virus and cells

Primary swine macrophage cell cultures were derived from pig peripheral blood and were prepared as described by Zsak et al. (1996).

Swine kidney cells (SK6) (Terpstra et al., 1990), free of BVDV, were used for virus titration. SK6 cells were cultured in Dulbecco's minimal essential medium (D-MEM) (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Atlas Biologicals, Fort Collins, CO).

CSFV Brescia (BICv) was derived from pBIC, an infectious cDNA clone described previously (Risatti et al., 2005a). Titration was performed in 96-well plates (Costar, Cambridge, MA) using SK6 cells. After 4 days in culture, viral infectivity was detected by an immunoperoxidase assay using the CSFV monoclonal antibody WH303 (Edwards et al., 1991) and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) (Risatti et al., 2003). Titers were calculated using the method of Reed and Muench (1938) and expressed as $TCID_{50}$ per ml. As performed, test sensitivity was $\geq$1.97 log 10 $TCID_{50}$ per ml.

2.2. RNA extraction and cDNA synthesis

Total cellular RNA was extracted from primary swine macrophage cell cultures infected at a multiplicity of infection of 1 (MOI = 1) $TCID_{50}$ per cell and from non-infected controls at 24 and 48 hpi. These time points represent the logarithmic phase of infectious virus assembly and release by infected cells and the peak of viral progeny yield, respectively (Fig. 1). RNA was extracted using a RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Contaminant DNA was removed by DNAse treatment using Turbo DNA-free (Ambion, Austin, TX). After DNAse treatment, genomic DNA contamination of RNA stocks was assessed by real-time PCR amplification targeting the porcine β-actin gene. Total RNA was quantified using a NanoDrop 1000 (Thermo Scientific, Waltham, MA). cDNA was synthesized with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) using random hexamer primers (Invitrogen, Carlsbad, CA) in a 100 μl reaction containing 1500 ng of total RNA.

2.3. Quantitative reverse transcription real-time polymerase chain reaction (qrt-PCR)

For gene expression quantification, first-strand cDNA was amplified by real-time PCR using *Power* SYBR® Green PCR Master Mix (Applied Biosystems) with primer pair sets described in Table 1. A 50 μl reaction contained 25 μl of *Power* SYBR® Green PCR Master Mix, 5 μL of cDNA, and 400 nmol/μl of each primer. Cycling conditions were as follows: activation of the AmpliTaq Gold® Polymerase at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min, with a melting curve analysis performed at the conclusion of the PCR assay. Real-time PCRs were run with an ABI 7900HT real-time PCR system (Applied Biosystems). Quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method (described in ABI PRISM® 7700 Sequence Detection System User Bulletin #2 (PN 4303859)), and was analyzed with ABI software: RQ Manager 1.2.

2.4. Statistical analysis

For each independent gene expression experiment ($n = 5$, performed with macrophages obtained from blood of different animals) a one-way analysis of variance (ANOVA) was used to compare the experimental exposure group to the unexposed control. ANOVA analysis was evaluated using the SigmaStat Windows 1.0 (Jandel Scientific, San Rafael, CA) software, using $p < .05$ for statistical significance.

3. Results

Growth kinetics of CSFV strain Brescia was performed in a multistep growth curve. Primary porcine macrophage cell cultures were infected at MOI = 1 $TCID_{50}$ per cell. Virus was adsorbed for 1 h (time zero) and samples were collected at times post-infection through 72 h. Virus titration was performed in SK6 cells. The logarithmic phase of infectious virus assembly and release by infected macrophages started between 8 and 24 hpi, with the peak of viral progeny yield observed at 48 hpi (Fig. 1).

To assess changes in cellular gene expression upon CSFV Brescia infection, primary porcine macrophage cell cultures were infected at MOI = 1 of $TCID_{50}$ per cell. Total cellular RNA was extracted from infected and mock-infected cells at 24 hpi (exponential growth) and 48 hpi (growth plateau). Prior to synthesis of cDNA, total RNA was treated with DNAse and tested for genomic DNA contamination by means of PCR (see Section 2). Steady state levels of mRNA accumulation were determined for 58 swine genes (Table 1) by means of qrt-PCR followed by melting curve analysis. Normalization of gene expression was carried out by assessing mRNA accumulation of a housekeeping gene (β-actin) in infected and mock-infected cell cultures. Relative quantities (RQ) of mRNAs were calculated by $2^{-\Delta\Delta Ct}$ method (see Section 2). The normalized mRNA expression level of a cellular gene in CSFV Brescia-infected cells was

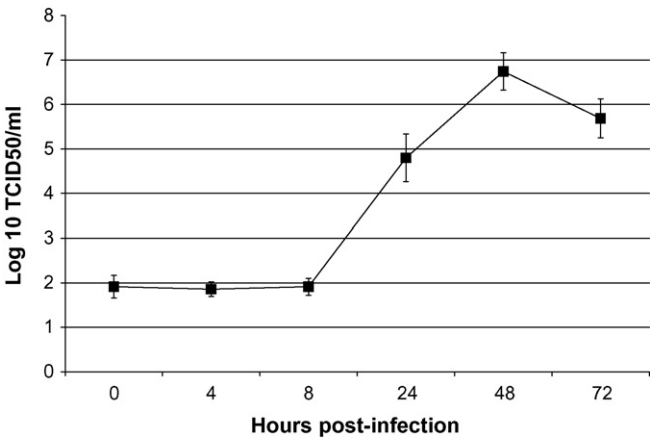

Fig. 1. Growth characteristics of CSFV Brescia (BICv) on swine macrophage cell cultures. Primary swine macrophage cell cultures were infected (MOI = 1). At times post-infection, samples were collected and titrated on SK6 cells for virus yield. Data are means and standard deviation of three independent experiments.

Table 1
Host genes analyzed and primer sets used in this study

Genes	Forward primer (5′ → 3′)	Reverse primer (5′→ 3′)
Cytokines		
IL-1α	GTGCTCAAAACGAAGACGAACC	CATATTGCCATGCTTTTCCCAGAA
IL-1β	AACGTGCAGTCTATGGAGT	GAACACCACTTCTCTCTTCA
IL-2	TCAACTCCTGCCACAATGTATAAGA	CTTGAAGTAGGTGCACCGTTTG
IL-4	GCCGGGCCTCGACTGT	TCCGCTCAGGAGGCTCTTC
IL-5	CGTTAGTGCCATTGCTGTAGAAA	CAAGTTCCCATCGCCTATCAG
IL-6	CTGGCAGAAAACAACCTGAACC	TGATTCTCATCAAGCAGGTCTCC
IL-7	GAGTGACTATGGGCGGTGAGA	GCGGGCGTGGTCATGA
IL-10	CGGCGCTGTCATCAATTTCTG	CCCCTCTCTTGGAGCTTGCTA
IL-12p35	CGTGCCTCGGGCAATTATA	CGCAGGTGAGGTCGCTAGTT
IL-12p40	AACTCTTCACGGACCAAATCTCA	GGTCCCGGGCTTGCA
IL-13	CCTGGAATCCCTCATCAACATC	AGGGCGCTCAGCATCCT
IL-15	GCTGTATCAGTGCAGGTCTTCCT	TTTCAGTATACAATGTGGCATCCA
IL-16	CGAAGACCCAGGTGCAAATAGT	CCGAAAGGTTGAGCGAGAAG
TNF-α	AACCTCAGATAAGCCCGTCG	ACCACCAGCTGGTTGTCTTT
TGF-β1	CCTGCAAGACCATCGACATG	GCCGAAGCTTGGACAGAATC
TGF-β2	TGTGTGCTGAGCGCTTTTCT	GAGCGTGCTGCAGGTAGACA
NCP-1	CGCCATCTCCTCCTGTACCA	GCAGTGACAATGGCAGCAAT
Chemokines		
IL-8	AAGCTTGTCAATGGAAAAGAG	CTGTTGTTGTTGCTTCTCAG
AMCF-1	GCTCGTGTCAACATGACTTCCA	GCCTCACAGAGAGCTGCAGAA
AMCF-2	CCACACCCGGGATTCATC	GGCTATCACTTCTGCCTTGGA
MCP-1	GCAGCAAGTGTCCTAAAGAAGCA	GCTTGGGTTCTGCACAGATCT
MCP-2	AAGACCAAAGCCGACAAGGA	TCATGGAATTCTGGACCCACTT
RANTES	AGCATCAGCCTCCCCATATG	TTGCTGCTGGTGTAGAAATATTCC
Toxicity response		
iNOS	GCGATGGGAAGCACGACTT	CATCTGGTAGCCGGCATACC
IL-1β converting enzyme		
ICE	GCCAAGAGGGAGCCTCAAG	CTCTGCTGACTTTTCTTTCCATAGC
Adhesion molecules		
ICAM	CACAGGCCGCCACTAACAA	GGTTCCATTGATCCAGGTCTTG
VCAM	GCTCCCAGGGATACGACCAT	ACTAGAGCAGGTCATGTTCACAGAA
Antiviral effect		
PKR	AAAGCGGACAAGTCGAAAGG	TCCACTTCATTTCCATAGTCTTCTGA
OAS	GAGCTGCAGCGAGACTTCCT	TGCTTGACAAGGCGGATGA
Mx1	GGCGTGGGAATCAGTCATG	AGGAAGGTCTATGAGGGTCAGATCT
Interferons and interferon regulatory factors		
IFN-α	TCTCATGCACCAGAGCCA	CCTGGACCACAGAAGGGA
IFN-β	AGTGCATCCTCCAAATCGCT	GCTCATGGAAAGAGCTGTGGT
IFN-γ	CGATCCTAAAGGACTATTTTAATGCAA	TTTTGTCACTCTCCTCTTTCCAAT
IRF-1	GCACCAGCGACCTGTACAACT	TCCTCATCTGTTGCAGCTTCA
IRF-3	CGCTTCTTGCCCCAACCT	TCCCACTCGTCGTCATTCG
IRF-6	GTGTACTGGTCTGGGCCATGT	GACCTTCTTTTGCCTCTCAATCA
IRF-7	CGCCTCCTGGAAAACCAA	CCCTGAGTTGTCCTGCAACA
IRF-9	GGCCCTGCCATCTGGAA	CAGGAACCTCCTCAAACTCAGTACT
Complement regulatory proteins		
CD46	GTGTCGTTGGCCCTAGTGTTC	GGCCGCATGCTTTCAAACT
Cytokine receptors		
IL-1R	TTGCTTTGCAGGTAATCATCAGA	TGCTGAACAAGGACACCACAGT
IL-2R-α	GTATGCGGGCTGGATGCT	TGCCTAAGCTGTTGCTATTCCAA
IL-6R	GGTACCATTGCCCACATTCC	GAACCTTAAGATGATGCCAATGC
IL-8R	CATCTTCCGTGAAGCCTACCA	TTGTATTGGCGCCCAGATC
IL-12R	CTGACTCAAAGCCGGGAAAC	ATCCATGTTGGAGGGTAAGTAACC
TNF-R	CCGCTGGTGATCTTCTTTGG	CGTTGGTAGCGGCAAGCT
TGF-β3R	TTCTCCACCGATTTTTCATGGT	AGCAGAGCTCCGATCACAAAC
DAP-12	GAGCCCAAATCAGGACAGTCA	GTATCCTGGTGGGAGCAGAAAG
NKG2D	AATCACAAGCAAATGTGGAGAAAA	AAACGGATCCCCATAGCTATAGC
Toll-like receptors		
TLR-1	TCCTGATCTTGCTGGATTCCA	TCCTTGGGCCATTCCAAA
TLR-2	GGCAAGTGGATTATTGACAACATC	ACCACTCGCTCTTCACAAAGTTC
TLR-3	CAAAACCAGCAACACGACTTTC	AATCATTACCAATCACACTTAAGCTGTTA
TLR-4	CCTCGGAGGCTGCTCTGA	GCGGATGCGAGGAATCAT
TLR-5	TGCTAGATGTTTCTGGCAATGG	GGCGAGAACCAAGGAGGAA
TLR-6	TCTCACAATCAGTTGCAGACAATCT	CATATGGGCAGGGCTTCAAA
TLR-7	CAAGCACTTGACAGCGATTCC	GGAAGGAGGCTGGAGTGATG
TLR-8	AAGGGCTGCAAAATCTGACTAAAA	AAACGCCCCATCTGTAATAGTCAT
TLR-9	TGTCCAGCCTACGAACTCTCAA	GTTGGGCTCGATGGTCATGT
TLR-10	ACTGCATGGAGAAAAGCTCTAAGTC	GAGGTGGTGGTGGCCAAAG

IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; NCP, neutrophil chemotactic protein; AMCF, alveolar macrophage chemotactic factor; MCP, monocyte chemotactic protein; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; PKR, protein kinase R; OAS, 2′-5′-oligoadenylate synthetase; Mx, interferon-inducible GTPases; IFN, interferon; IRF, interferon regulatory factor; CD, cluster of differentiation; DAP, transmembrane adaptor protein; NKG, natural killer cell receptor; TLR, toll-like receptor.

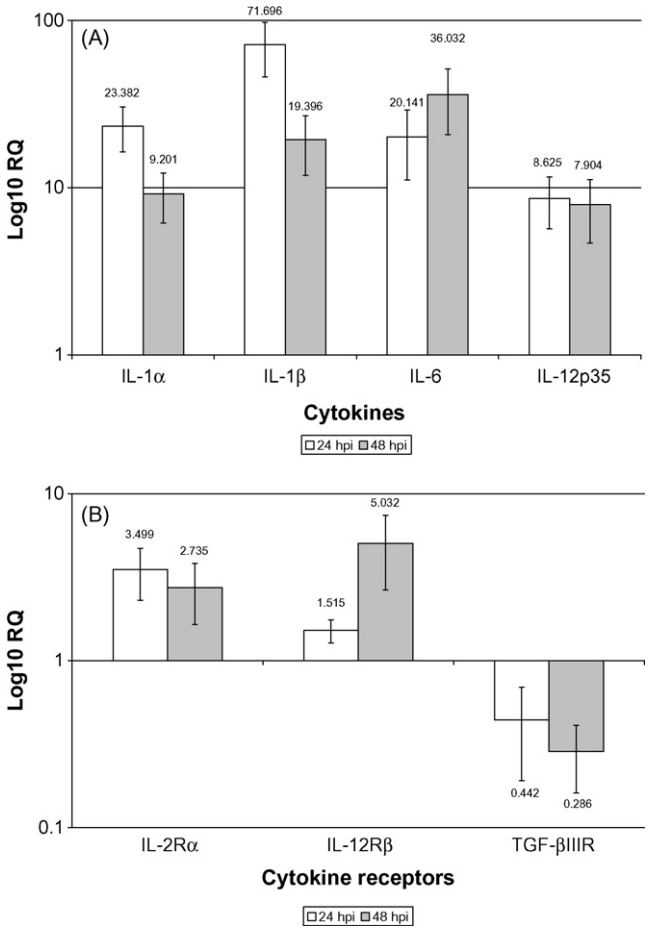

Fig. 2. Cytokines (A) and cytokine receptors (B) gene expression changes in peripheral blood derived macrophages after infection with CSFV strain Brescia for 24 and 48 h. Gene expression quantification was assessed by quantitative reverse transcription real-time polymerase chain reaction (qrt-PCR). Relative quantities (RQ) of mRNA accumulation were estimated by $2^{-\Delta\Delta Ct}$ (see Section 2).

considered significant when it departed from its level in uninfected cells threefold, in either direction (Brukman and Enquist, 2006). The approach enabled the identification of 18 genes differentially expressed in primary porcine macrophages infected with CSFV Brescia (Figs. 2–4).

The highest sustained level of mRNA accumulation upon infection was observed for cytokine genes. Expression analysis of 17 cytokine genes (Table 1) showed significant up-regulation of IL-1α, IL-1β, IL-6, and IL-12p35 at 24 and 48 hpi (Fig. 2A). IL-1β expression reached a 71-fold increase by 24 hpi and a 20-fold increase by 48 hpi, respectively (Fig. 2A), the highest mRNA accumulation observed in this study. Cytokine receptors IL-2Rα and IL-12Rβ mRNA levels, on the other hand, showed a moderate accumulation at 24 and 48 hpi, respectively, with TGF-βIIIR expression down-regulated up to threefold by 48 hpi (Fig. 2B). The expression of other cytokine receptors IL-1R, IL-6R, IL-8R, TNF-R, DAP-12, and NKG2D were not affected by CSFV Brescia infection at the tested time points (Fig. 2B). CSFV Brescia infection significantly affected the expression of several chemokine genes. Up-regulation of chemokines IL-8, AMCF-1, AMCF-2, MCP2, and RANTES was observed at 24 or 48 hpi, with changes in the level of expression ranging between 3- and 12-fold over mock-infected control cells (Fig. 3). MCP-1 expression was not affected at the tested time points.

Changes in the expression of type I interferon were observed in infected cultures. INF-α and INF-β, but not INF-γ, mRNA levels

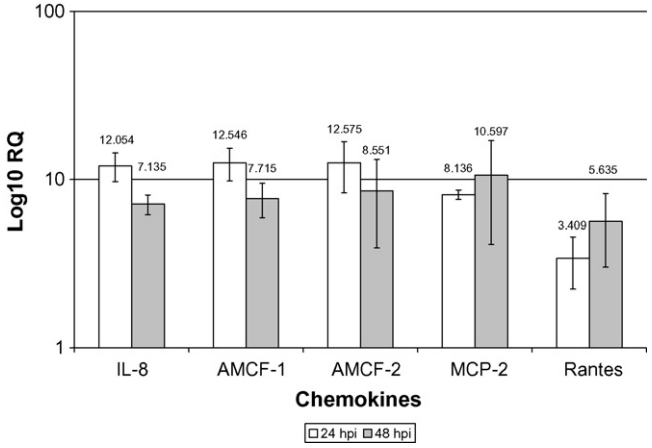

Fig. 3. Chemokine gene expression changes in peripheral blood derived macrophages after infection with CSFV strain Brescia for 24 and 48 h. Gene expression quantification was assessed by quantitative reverse transcription real-time polymerase chain reaction (qrt-PCR). RQ of mRNA accumulation were estimated by $2^{-\Delta\Delta Ct}$ (see Section 2).

were affected upon CSFV Brescia infection. While INF-α mRNA levels increased towards 48 hpi, INF-β mRNA levels accumulated by 24 hpi and decreased by 48 hpi (Fig. 4A). Interestingly, mRNA levels of interferon regulatory factors (IRFs) 1, 3, 6, 7, and 9 or antiviral

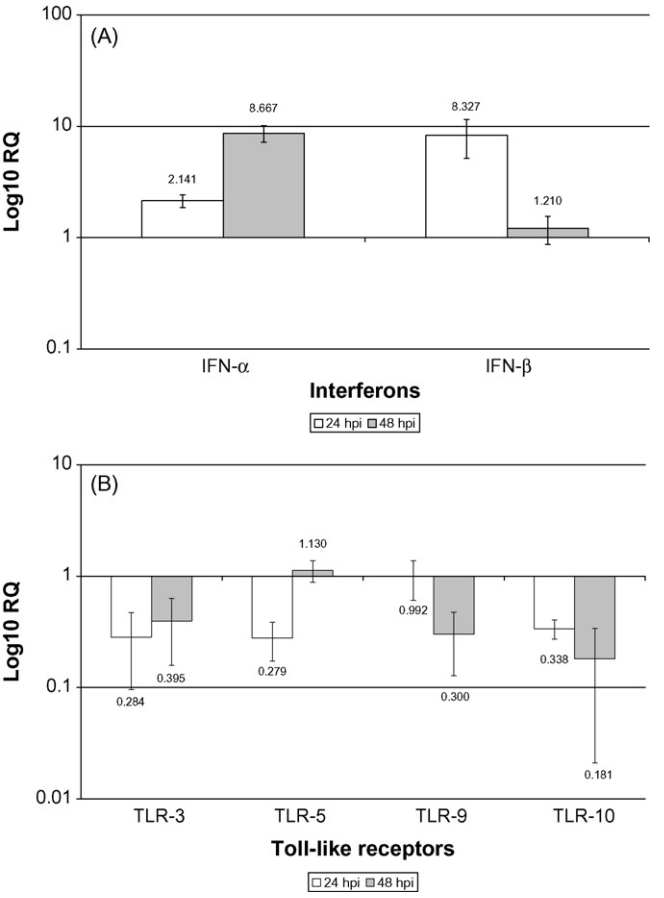

Fig. 4. Type I interferon (A) and toll-like receptors (B) gene expression changes in peripheral blood derived macrophages after infection with CSFV strain Brescia for 24 and 48 h. Gene expression quantification was assessed by quantitative reverse transcription real time polymerase chain reaction (qrt-PCR). RQ of mRNA accumulation were estimated by $2^{-\Delta\Delta Ct}$ (see Section 2).

effector genes like PKR, OAS, and Mx1 remained unchanged at 24 and 48 hpi.

As with type I interferon response, expression of toll-like receptors (TLRs) is modulated rapidly in response to pathogens. We observed that TLR3, TLR5, TLR9 and TLR10 expressions were affected by CSFV Brescia infection (Fig. 4B). Up to fivefold down-regulation of TLR10 expression was observed by 48 hpi. Expression of TLR1, TLR2, TLR4, TLR6, TLR7, and TLR8 genes remained unmodified at both time points tested here.

4. Discussion

To further characterize the nature of host cell responses to infection by virulent CSFV Brescia, we used qrt-PCR to assess changes in expression of 58 cellular genes involved in modulation of the host immune response in swine macrophages. Peripheral blood-derived swine macrophages are a CSFV primary target cell *in vivo* and seem to play a central role in the outcome of the infection (Gomez-Villamandos et al., 2001; Sanchez-Cordon et al., 2002, 2003; Summerfield et al., 1998).

Out of 17 cytokine genes analyzed, (Table 1) four, including IL-1α, IL-1β, IL-6, and IL-12p35, showed significantly higher levels of expression upon infection (Fig. 2A). Changes in the expression of these cytokines due to CSFV infection have been reported previously. Transcriptional response studies of swine macrophages infected with the Brescia strain, by means of microarrays, revealed increased accumulation of IL-1β mRNA (Zaffuto et al., 2007). Increased levels of IL-1α have been observed *in vivo* in lymphoid tissues of pigs (Sanchez-Cordon et al., 2005a). *In vitro*, swine endothelial cells (Bensaude et al., 2004) infected with virulent CSFV strain Alfort 187 showed increased expression of cytokines, including IL-1α. Pro-inflammatory cytokines, cell adhesion molecules and blood coagulation factors induced in endothelial cells likely contribute to the appearance of vascular lesions characteristic of the disease (Gomez-Villamandos et al., 2000; Bautista et al., 2002; Sanchez-Cordon et al., 2002, 2003). IL-6, as well as IL-1, is also able to activate endothelial cells leading to the release of IL-8 and MCP, expression of adhesion molecules, and recruitment of leukocytes to inflammatory sites (Gabay, 2006; Lipsky, 2006). Both cytokines are potent inducers of vascular permeability and mediate pathologic changes including high fever, coagulation defects, and bleeding observed in infections with some single-stranded RNA viruses (Bray, 2005). Expression of IL-6 increased in CSFV-infected macrophages *in vitro* (Fig. 2A) and *in vivo* (Sanchez-Cordon et al., 2005a), but not in porcine bone marrow-derived dendritic cells (DC) infected with the Brescia strain (Carrasco et al., 2004). Likely, macrophages might be an important source of mediators, like IL-6 and IL-1, affecting the vascular dysfunctions observed in swine during CSFV Brescia infection.

Whereas IL-6, IL-1α, and IL-1β may play a direct role in the altered haemostatic balance observed during CSFV infection, IL-12 modulation may induce a different effect. Interleukin-12p70 (IL-12p70) is the main immunoregulatory cytokine that governs Th1 immune response polarization (Dobreva et al., 2008). IL-12p70 is a heterodimeric protein consisting of 40-kD (p40) and 35-kD (p35) subunits (Wolf et al., 1991). These subunits are encoded by two separate genes whose expression is independently regulated at the transcriptional level (Aste-Amezaga et al., 1998). Cells, like phagocytes and DCs that produce IL-12p70 and IL-23 heterodimers (D'Andrea et al., 1992; Macatonia et al., 1995), secrete a monomer of the p40 chain at an excess of several to 1000-fold above the heterodimer (Aste-Amezaga et al., 1998). Plasmacytoid DCs obtained from blood and secondary lymphoid organs of CSFV-infected pigs at an early time post-inoculation were activated and expressed both INF-α and IL-12 (Jamin et al., 2008). In this study we observed accumulation of IL-12p35 chain mRNA over IL-12p40 chain mRNA (Fig. 2A) that may result in a decreased production of active IL-12p70 by CSFV-infected macrophages. Potent immunosuppressive agents like Cyclosporine-A (CyA) and FK506 have the ability to suppress the production of Th1 cytokines via interleukin IL-12p40 suppression (Ma et al., 2007). Whether the differences in IL-12 expression we observed here are a function of macrophage activation or have an effect on suppression of Th1 response will require additional studies.

Contrary to the observations showing increased levels of TNF-α expression in spleen macrophages of animals infected with Alfort strain (Sanchez-Cordon et al., 2005b), or in pig lymph nodes and in cultured pulmonary alveolar macrophages infected with strain SNUVR1998 (Choi et al., 2004), our study demonstrated that steady state levels of TNF-α remained unchanged upon infection. Interestingly cell-type specific regulation of TNF-α expression has been observed between conventional and plasmocytoid DCs obtained from blood and secondary lymphoid organs of infected pigs at early times post-inoculation (Jamin et al., 2008).

The slight accumulation of IL-2R mRNA observed by 24 hpi coincides with a down-regulation of TGF-β IIIR (Fig. 2B). While IL-2 mediates signaling through IL-2R, stimulating a set of complex signal transduction pathways resulting in cell proliferation (Minami et al., 1993), signaling through TGF-β IIIR–Smad results in inhibition of cellular proliferation, enhancement of matrix accumulation, and suppression of inflammation (Blobe et al., 2001). The putative shift we have observed, towards induction of cell proliferation suggested by IL-2R up-regulation coupled with TGF-β IIIR down-regulation, may stimulate virus replication. Stimulation of PBMCs by IL-2, the ligand for IL-2 receptor, induces replication of a variety of HIV strains, including primary isolates (Kinter et al., 1995). In this study we have observed increased levels of IL-12R mRNA by 48 hpi (Fig. 2B). IL-12 exerts its functions by binding to specific cell surface receptors and signaling through the Jak-STAT pathway (Wang et al., 2000). Signaling through IL-12R results in the production of TNF-α and INF-γ (Lodoen and Lanier, 2006). However, the expression of both cytokines remained unmodified at the time points tested here. Whether the undetected changes in TNF-α and INF-γ expression in macrophages is a function of IL-12p70 down-regulation rather than suppression of IL-12 receptor signaling remains to be determined.

The elevated levels of IL-1α, IL-6, IL-8, and RANTES mRNAs observed in infected swine macrophages (Figs 2A and 3) and the increased expression of IL-1α, IL-6, IL-8, and procoagulant factors in infected swine endothelial cells (Bensaude et al., 2004), together with mast cell degranulation and activation of the complement system in infected swine (Gomez-Villamandos et al., 2000), may account for the vascular dysfuntions observed *in vivo*. Vascular dysfunctions resulting in plasma leakage and derangements of hemostasis, as observed in Dengue virus infections (Bosch et al., 2002), seem to be mediated by cytokines, including IL-8, RANTES, and IL-6 released from infected target cells (Suksanpaisan et al., 2007; Huang et al., 2000; Ubol et al., 2008). IL-8, for example, destroys tight-junction and cytoskeleton reorganization, resulting in increased vascular permeability (Talavera et al., 2004), and RANTES stimulates trans-endothelial migration of inflammatory cells (Appay and Rowland-Jones, 2001; Middleton et al., 2002). Likely contributing to vascular permeability, MCP-2 (monocyte chemoattractant protein 2), a C–C chemokine (CCL8), is up-regulated during the infection (Fig. 3), and like RANTES, signals through CCR5, CCR2, and CCR3 receptors (Hellier et al., 2003).

Steady state levels of AMCF 1 and 2 (alvelolar macrophage-derived neutrophil chemotactic factor 1 and 2), an IL-8 homologue (Ledger et al., 2004), mRNAs were increased (Fig. 3). These chemokines are specific neutrophil chemoattractants both *in vitro* and *in vivo* (Goodman et al., 1992), resembling functions of CXC

chemokines that mediate an increase in circulating neutrophils derived from bone marrow (Burdon et al., 2008).

A temporal pattern of accumulation of type I interferon mRNAs was observed upon infection of swine macrophages with Brescia strain. INF-α transcripts increased more than eightfold by 48 hpi, while the opposite trend was observed for INF-β mRNA, with a sevenfold decrease by 48 hpi (Fig. 4A). *In vitro* studies have shown that CSFV infection precludes the synthesis of type I interferon by porcine cells, including macrophages (Ruggli et al., 2003), myeloid dendritic cells (Carrasco et al., 2004), and endothelial cells (Bensaude et al., 2004), but the infection induces production of IFN by porcine plasmocytoid dendritic cells (Balmelli et al., 2005). The lack of IFN-α/β induction in CSFV-infected cells has been associated with loss of IRF 3 (La Rocca et al., 2005; Bauhofer et al., 2007). In both cases it was demonstrated that the effect was mediated by CSFV N^{pro} protein. La Rocca et al. first concluded that CSFV infection precluded IRF3 expression, but Bauhofer et al. subsequently showed that loss of IRF3 was due to degradation by the proteosome. Noteworthy, these effects were observed in a porcine kidney cell line (PK15 cells) and in both cases amounts of IRF3 was readily detected in infected cells at the last time point assessed (32 hpi). The effect of the observed IRF3 levels late after infection on the expression of type I interferon was not established. Furthermore, Bauhofer et al., observed that IRF7 was not down-regulated by CSFV infection of PK15 cells. In certain cell types IFN-α/β induction is essentially IRF7 dependent (Honda et al., 2005b). *In vivo*, IFN-α production, not IFN-β, is induced in the acute phase of the infection with virulent strains and can be readily detected in serum of infected swine (2–3 days post-infection) even before the viremic phase (4–5 days post-infection) (Summerfield et al., 2006). Our observations coincide with the *in vivo* scenario observed by Summerfield et al. (2006).

Interestingly, absence of IFN activity in supernatants but presence of IFN-α and IFN-β mRNAs has been observed in blood-derived swine macrophage cultures treated with LPS (Knoetig et al., 2002). This observation was made while studying LPS-induced impairment of CSFV infection in macrophages and concluded that IFN levels in culture supernatants may have been below the limits of detection. In our study total RNA was extracted from infected and mock-infected cells at similar time points (24 and 48 hpi). Real-time RT-PCRs were run in parallel and mRNA quantifications were normalized relative to non-infected macrophages. In all cases, similar Ct values were observed for IFN-α and IFN-β at both time points in non-infected cells, suggesting that cultures did not contain type-I interferon activators. Hence, the observed differences were due to CSFV Brescia infection.

In this study, we have observed significant reduction of TLR-3, 5, 9, and 10 expressions (Fig. 4B). Although the functions and natural ligands of swine TLRs are still unclear, these molecules are highly conserved across vertebrates suggesting similar activities. TLRs are pattern-recognition receptors (PRRs) that recognize structurally conserved molecules derived from microbes (Akira, 2006; Beutler, 2007; Meylan et al., 2006). Expression of TLRs is modulated rapidly in response to pathogens (Akira, 2006). TLR3 detects double-stranded (ds) RNA. TLR9 recognizes unmethylated DNA with CpG motifs. TLR5 recognizes flagellin from flagellated bacteria. TLR10 remains the only orphan member among the human TLRs. No natural or synthetic ligand has been assigned to TLR10 (Hasan et al., 2005). The functional significance of the TLR expression changes observed here remains unclear. Upon binding with specific ligands TLRs are potent activators of cellular innate immune signaling pathways. In mice, for instance, type-I interferon response can be triggered in many cell types by cytosolic detection of viral infection (mainly DNA viruses), or in differentiated plasmacytoid dendritic cells, by the toll-like receptor 9 (TLR9) subfamily, which generates signals via the adaptor MyD88 to elicit robust IFN induction (Honda et al., 2005a). On the other hand, signaling through TLR3 is MyD88-independent and generates signal via TRIF (TIR-domain-containing adapter-inducing interferon-β) leading to IRF3 and NF-κB activation (Zhang et al., 2007). TLR3-deficient mice are susceptible to EMCV (Encephalomyocarditis virus), displaying impaired induction of pro-inflammatory cytokines and chemokines and a significantly higher viral load in the heart compared to wild-type mice following infection with EMCV (Hardarson et al., 2007). In mice and humans, TLR5 signaling is activated by its ligand, flagellin. TLR5 signals through MyD88 and elicits the production of inflammatory cytokines (Kawai and Akira, 2006). Interestingly, it has recently been shown that systemic administration of flagellin protects mice against chemical, bacterial, viral, and radiation challenge (Vijay-Kumar et al., 2008). Didierlaurent et al. (2008) have demonstrated that alveolar macrophages isolated after resolution of influenza infection have impaired NF-κB nuclear translocation to TLR ligation, which is associated with reduced subsequent acute inflammation and cell recruitment. The reduced neutrophil recruitment correlates with a higher and prolonged respiratory bacterial load. Thus, down-regulation of TLRs might have an important immunosuppressive effect.

The transcriptional profile of 58 genes involved with mechanisms of the innate immune response of swine macrophages shown here was aimed to identify mechanisms underlying the pathogenesis of virulent CSFV strain Brescia infection. Host genes identified here, showing altered transcriptional activity, are involved with signaling pathways relevant to the induction of harmful inflammatory responses and/or modulation of immune response observed during infections with highly virulent CSFV. The complex patterns of gene expression observed here and by others, and the potential relationships inferred from those observations warrant further assessment of the role of those genes during infection in swine.

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